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TITLE: Quantum-Dot-Based Automated Screen of Sentinel Lymph Nodes for Metastatic Breast Cancer

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14. ABSTRACT:

We are developing an automated technique for scanning lymph nodes for small numbers of breast cancer cells using a mouse model. This technique involves the use of standard primary antibodies for tumor specific antigens and quantum-dot conjugates in place of chemical fluorophores combined with exhaustive confocal z-sectioning and computer analysis. We have verified that the quantum dot conjugates using antibodies to Brst and cytokeratin are functional in frozen sections from actual tissue but will have to do additional work to reliably identify cancer cells in an automated way. The primary problems to still be solved involve reducing antibody background. Once these have been solved, we expect to be able to test our automated approach for sensitivity against standard histological methods for detecting breast cancer cells in lymph nodes.

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Breast Cancer

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INTRODUCTION:

This project is a Breast Cancer Concept Award aimed at testing whether breast cancer cells can be detected sensitively in lymph nodes through the use of quantum dot conjugates combined with confocal image acquisition and automated analysis. Since exhaustive z-sectioning by confocal microscopy is likely to require prolonged exposure to extremely high light intensities, which will likely produce severe photobleaching of conventional fluorophores we proposed the use of quantum dots. Our immediate aims were: 1) to test that quantum-dot conjugated antibodies could be used to detect breast cancer cells in cell culture and in tumors (where antibody penetration could be an issue) and 2) whether automated routines could be developed to detect cancer cells in microscopic images. The goal was to test the feasibility of these elements and if possible to create and test a fully automated technique to find small numbers of breast cancer cells in confocal images from sections spanning the entire thickness of a lymph node. This study was performed using mouse tissue culture cells and lymph nodes extracted from a mouse model for breast cancer. We find that the use of quantum dot conjugated antibodies is fully feasible for detection of cancer cells in lymph nodes as is confocal imaging of histological sections of lymph nodes stained with quantum-dot conjugated antibodies. However, while we found visual identification of tumor cells (identified by immunofluorescent stain for the Brst2 antigen or for cytokeratin) relatively easy, we did not succeed in developing robust computerized image analysis procedures. This was largely due to heavy background staining by the antibodies which created a high risk of false positives in our computer analysis algorithms which depended heavily on detection of high fluorescent intensities. The approach seems promising however provided that there is low background in non-cancer cells.

BODY:

Statement of Work Task 1:

Antibodies against two antigens found in human breast cancer cells and used for diagnostic purposes (cytokeratin and Brst2) were used to stain breast cancer cells of mouse origin and lymph nodes extracted from a mouse model of breast cancer. Monoclonal antibodies against cytokeratin and Brst2 were used which show cross-species reactivity with the mouse proteins. Secondary antibodies were purchased conjugated to biotin and staining was visualized using avidin conjugated to the conventional dye Cy3 or to quantum dots (purchased from Quantum Dot Corporation; emission peak at 605 nm). No difficulty was found visualizing the cytokeratin or Brst2 in cancer cells in culture with either quantum dots or conventional dyes (described more fully in the October 1, 2004 annual report). However conventional dyes bleached rapidly upon prolonged exposure to laser illumination (required for the prolonged confocal imaging required to acquire z-stacks through very thick sectioins) while quantum dots proved completely resistant to photobleaching.

Detection of cancer cells in frozen sections of lymph nodes from mice with breast cancer proved possible using an identical approach with either quantum dots or conventional fluorophores. This was partly staining of the cell surface of normal cells but appeared in part to be autofluorescence in the lymph nodes. Cytokeratin staining of tumor cells could be readily recognized by the distinctive pattern of fibers and the distinctive morphology of these cells. However computer identification of tumor cells depends on recognizing simple intensity differences. Since the goal of our study was to recognize isolated single tumor cells, an irregular pattern of background fluorescence (as we saw) greatly reduced the potential for computer analysis. This could be solved either by reducing the background (less fluorescence of non-tumor cells) or by greatly increasing the signal (more fluorescence in tumor cells). The antibodies we used were optimized for diagnostic use in human cells. While they were reported to have cross-reactivity to mouse and clearly could detect the mouse antigens, they may have been much less sensitive than they would have been in human cells (thus reducing signal as compared to background). That this may be the case is suggested by the very intense staining often observed with antibodies against cytoskeletal proteins. While the current study was conducted in non-human cells as required by the terms of the award, we are considering study of human-originated tissue in the future.

In summary, we obtained equivalent results staining either cells or tissue with antibody conjugated to conventional fluorophores or quantum dots. Staining was of sufficient quality to identify breast cancer cells visually in mouse lymph nodes or in tissue culture. However significant background staining was found with

either conventional fluorophores or quantum dots in lymph nodes (but not in culture). It is possible this is related to the use of human diagnostic tools in mice.

Statement of Work Task 2:

Confocal optical sectioning of thick sections of lymph nodes presented little problem with quantum dots (0.5 micron optical sections of 50 micron physical sections). However photobleaching was severe when conventional fluorophores were used if a large number of optical sections were acquired. Specifically, there was no problem with initially acquired sections, however significant photobleaching was seen towards the end of the z-stack due to the prolonged laser illumination required. In contrast photobleaching was not an issue with quantum dots. Thus acquisition of high-resolution information through a thick slice is facilitated by the use of quantum dots. In tests we found we could get images of acceptable quality from a 150 micron thick slice although image quality was noticably degraded at large distances from the coverslip. The major problem was the complexity of the fluorescence patterns in the image (noted above). Additionally, acquiring large z-stacks was very time-consuming as compared to acquiring single images with a wide-field scope. Since human lymph nodes are much larger than mouse lymph nodes, this could be a serious obstacle but could be speeded by using both low numerical aperture objectives (and thus thick optical sections) and special confocal optics (such as spinning disk configurations).

To analyze these images we constructed software written in C which first background corrects the image locally using a median filter and then thresholds the image to identify objects (defined as collections of above-threshold pixels). We produced both 2-D and 3-D versions of the code but had difficulty processing the images since thin fluorescent regions of high intensity (apparently lying between cells) overlapped somewhat making regions with overall a large number of pixels (thus meeting the criteria of cells). This problem was due primarily to insufficient contrast in the image (as described previously) rather than any problem with the image processing algorithm. One important consideration is that our 2-D code proved very fast. The 3-D code could not run with an entire Z-stack in computer memory (RAM) at once (on a Dell 2 Ghz PC with 2 GB memory) but could only work on limited numbers of slices at a time. This significantly degraded performance.

The overall image processing approach seems to be sound in principle but requires images with high contrast between cancer cell staining and staining of other objects. Additionally, processing of 3-D images efficiently requires significant computer capacity and would probably benefit from algorithm development by someone trained in computer science or in engineering.

KEY RESEARCH ACCOMPLISHMENTS:

1) Immunofluorescence using quantum dots can successfully visualize tumor-specific antigens in breast cancer cells.

2) Quantum dot conjugates penetrate frozen sections of lymph nodes as well as conventional fluorophore conjugates.

3) Quantum dot conjugates appear superior to conventional fluorophores when confocal images are acquired from very thick samples due to photobleaching resistance.

4) Finding fluorescent objects in 3-D data sets in an automated manner is feasible provided the fluorescent objects are sufficiently brighter than all background objects.

REPORTABLE OUTCOMES:

We intend to continue this research and anticipate it will result in a publication once we can resolve the issue of background staining.

The following personnel were supported by the award. Archana Srivastava, Rohit Kumar.

CONCLUSIONS:

We have established that quantum dots show comparable performance to conventional fluorophores for detection of tumor cells in either cell culture or in lymph nodes of mice. Under conditions of prolonged illumination (as required for confocal acquisition of large z-stacks) quantum dots have considerably superior performance to chemical fluorophores. We have not found any evidence of penetration problems by the quantum dots which was our initial major concern (due to the 5 – 15 nm size of the dots). Computer analysis of the entire thickness of a lymph node appears feasible in principle. We had problems but that appears to only be due to difficulties with high background antibody staining (equally with chemical fluorophores and quantum dots) which may have been specific to our model system and thus can probably be overcome. Thus it appears technically feasible to examine human lymph nodes in a highly automated way for small numbers of cancer cells, although more work will be required. This has potential to detect breast cancer metastasis more sensitively than currently and thus save lives.

REFERENCES:

n/a

APPENDICES:

n/a

SUPPORTING DATA:

n/a